JURNAL FARMASI SAINS DAN KOMUNITAS
Journal of Pharmaceutical Sciences & Community

p-ISSN 1693-5683; e-ISSN 2527-7146

Vol. 19, No. 1, May 2022, pp. 1-7

Research Article

Inhibitory Activity of *Parsea americana* Mill. Peels Extract and Fraction Containing Phenolic Compound Against *Staphylococcus aureus* ATCC 25923

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https://doi.org/10.24071/jpsc.003005

J. Pharm. Sci. Community, 2022, 19(1), 1-7

Article Info

Received: 05-12-2020
Revised: 06-04-2021
Accepted: 06-05-2021

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Keywords:
inhibitory activity; *Parsea americana* Mill. peels; phenolic content; *Staphylococcus aureus*

ABSTRACT

Parsea americana Mill. is a natural resource that has been studied for its antibacterial properties. The pulp, peel, and seed of *Parsea americana* Mill. have potential as an antibacterial agent. This study aimed to determine the inhibitory activity and phenolic content of *Parsea americana* Mill. peels extract and fraction against Staphylococcus aureus ATCC 25923. *Parsea americana* Mill. was macerated with 96% ethanol and then fractionated with *n*-hexane, ethyl acetate, and methanol solvent. Determination of the fraction that has the greatest antibacterial activity against *S. aureus* was carried out using the Kirby Bauer method. The total phenolic content in the extract and fraction was calculated as gallic acid equivalent (GAE) using the Folin-Ciocalteu method spectrometrically. Antibacterial activity test of the 96% ethanol extract, ethyl acetate fractions, and methanol fractions at a concentration of 10% w/v showed activity with a measurable inhibition zone. On the other hand, the *n*-hexane fraction showed no inhibition zone. The highest inhibition zone was the ethyl acetate fraction with approximately 8.33 ± 0.58 mm. The ethyl acetate fraction of *Parsea americana* Mill. resulted in 536.26 ± 14.29 mg GAE/g fraction. The conclusion was that the ethyl acetate fraction had the highest total phenolic content and was the most active fraction in inhibiting the growth of *Staphylococcus aureus*.

INTRODUCTION

Avocado, known as the Latin name *Parsea americana* Mill., is a fruit that has many nutrients and the public widely consumes it. Besides that, avocado is widely used in mixtures of cosmetic ingredients. Utilization of avocado pulp is not proportional to the use of its avocado peels, so that the avocado peels are often not used (Fauziah *et al*., 2016). *Parsea americana* Mill. is one of the most researched natural resources for its antibacterial potential (Efendi, 2019).

Parsea americana Mill. is a fruit cultivated in most tropical and subtropical countries. Avocado is a member of the Lauraceae family with the genus and species identified as *Parsea americana* (Kavaz and Ogbonna, 2019). *Parsea americana* Mill. peel, fruit, and leaves are commonly used commonly used in the treatment of several diseases such as stomach pain, menorrhagia, diarrhea, diabetes, and hypertension (Cardoso *et al*., 2016). *Parsea americana* Mill. leaves, peel, and seed have biological activities that are scientifically proven (Amado *et al*., 2019). In several studies, *Parsea americana* Mill. leaves were demonstrated to have antioxidant and antibacterial activity. The total phenolic content of different avocado varieties were previously identified. The avocado peel extract has been shown to have an antibacterial activity against both gram-positive

and gram-negative bacteria (Amado *et al*., 2019; Carpena *et al*., 2011).

Staphylococcus aureus is a gram-positive bacteria typically found with other commensals in human skin and mucous but also can cause severe infections (Schmidt *et al*., 2015). Moreover, *Staphylococcus aureus* is one of the causes of post-operative wound infection, toxic shock syndrome, and food poisoning (Bachir and Benali, 2012). Antibacterial activity of plant extract is caused by the presence of phytochemicals such as terpenoids, essential oils, alkaloids, lectins, polypeptides, polyphenolics, and phenol substances (Cardoso *et al*., 2016). The phenolic compounds are some of the compounds that have pharmacological activities including antioxidants which are anti-aging agents and important for the prevention and treatment of degenerative diseases, cancer, and immune system disorders. Furthermore, phenolic compounds were also reported to have activity to retain antibacterial therapy in certain bacterial strains such as *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Pseudomonas spp., Escherichia coli, and Klebsiella pneumoniae* (Carpena *et al*., 2011; Ogundare and Oladejo, 2014). The phenolic compounds in the *Parsea americana* Mill. peel extract are higher than their content in pulp and seed extract (Amado *et al*., 2019). Fractionation is the process of separating secondary metabolite compounds according to their polarity level. The fractionation process is done sequentially, starting from the non-polar solvent to the polar solvent (Purwanto, 2015). Fractionation will show a different total phenolic yield. Determination of phenolic content is a reflection of antibacterial activity (Amado *et al*., 2019; Carpena *et al*., 2011). Previous research was limited to the measurement of total phenolic extract and there have been no studies related to total phenolic test on *Parsea americana* Mill. peel fractions. This research aimed to determine total phenolic content of the *Parsea americana* Mill. peel extract and fractions with the Folin-Ciocalteau method (Nurcahyanti *et al*., 2011). Using these results, the antibacterial activity of the extract and fractions was determined by the Kirby-Bauer method (Carpena *et al*., 2011).

METHODS

Materials and Chemicals

The peels of *Parsea americana* Mill. were collected from some markets of Demak, Jawa Tengah, Indonesia. *Staphylococcus aureus* ATCC 25923 isolates were obtained from the Institute for Health and Calibration Laboratory Yogyakarta. Media for bacteria were Brain Heart Infusion (Oxoid) and Mueller Hinton Agar (Oxoid). Chemicals were included : NaCl 0.9% sterile (Widatra), barium chloride (Merck), sulphuric acid (Merck), ethanol 96% technical solvent (Bratachem), *n*-hexane technical solvent (Bratachem), ethyl acetate technical solvent (Bratachem), methanol technical solvent (Bratachem), paper disk (Oxoid), vancomycin disk (Oxoid), gallic acid (Merck), the Folin-Ciocalteu's phenol reagent (Merck), methanol pro analysis (Merck), sodium carbonate anhydrous (Merck), and bi-distilled water.

Extraction and Fractionation

Parsea americana mill. peels were dried in a dryer cabinet at 50^{o}C for five days then mashed by blending and sieved using mesh 60 to obtain a powder of the fruit peel. The dry powder of *Parsea americana* Mill. peels (200 g) was extracted by maceration method using 2,000 mL ethanol 96% (1:10) then stirred until homogenous. Next, it was allowed to stand for 24 hours while stirred every 4 hours and the solvent was changed three times. The macerate was filtered and concentrated using a rotary evaporator (Wulandari *et al*., 2019). The *Parsea americana* Mill. extract was suspended in warm water and fractionated using the liquid-liquid extraction method with *n*-hexane, ethyl acetate, and methanol as solvent, respectively (Efendi, 2019).

Total Phenolic Content

The total phenolic content of extract and fractions was determined using the Folin-Ciocalteu method as described by Nurcahyanti *et al*. (2011). The sample was prepared by dissolving 50 mg of the *Parsea americana* Mill. extract and fractions using methanol in 10 mL to obtain concentration of 5mg/mL. A total of 200 µL *Parsea americana* Mill. extract and fractions solution was put in a test tube filled with 3 mL of bi-distilled water. Next, 0.4 mL of a Folin-Ciocalteau reagent was added to the liquid mixture, then incubated for 5 minutes at 25^{0}C. As much as 4 mL of 7% sodium carbonate was added to the liquid mixture, and then 10 mL of bi-distilled water were added. Next, the liquid mixture was shaken gently. Absorbance was measured with a ultraviolet (UV)-Visual Spectrophotometer (Shimadzu) at 750 nm using the prepared blank after being incubated for 120 minutes at 25^{0}C.

A calibration curve was established using gallic acid (0.09 to 0.30 mg/mL) as the standard reference. Total phenolic content of extract and fraction *Parsea americana* Mill. was revealed as

gallic acid equivalent (GEA) in milligrams per gram extract or fractions (Nurcahyanti *et a*l., 2011).

Media Preparation

Brain Heart Infusion (BHI) media was prepared by adding 4.44 g of BHI powder into 120 mL of distilled water and then stirring until dissolved and homogeneous. The mixture was then sterilized at 121°C for 15 minutes using an autoclave (All American) (Yunus *et al*., 2017).

Mueller Hinton Agar (MHA) media was prepared by adding 38 g of MHA powder in 1,000 mL of distilled water, then boiled and stirred for one minute until completely dissolved and homogeneous. After that, the media was sterilized by autoclaving for 15 minutes at 121°C (Utomo *et al*., 2018). When the MHA solution was warm enough, then the media was poured aseptically into petri dishes, and allowed to stand at room temperature until solidified. Then, it was stored at 4°C (in the refrigerator) if not used immediately.

McFarland 0.5 Standard Preparation

McFarland 0.5 standard was prepared by mixing 0.05 mL of 1.175% barium sulphate ($BaSO_4$) and 9.95 mL 1% sulphuric acid. The turbidity was used as the standard of the test bacterial suspension and equivalent to bacterial density of 1.5 x 10^8 CFU/mL (Zepata and Ramirez-Arcos, 2015).

Inoculum Preparation

Inoculum preparation of *Staphylococcus aureus* were determined by using a modified method according to Marfuah *et al*. (2018). *Staphylococcus aureus* from stock culture was taken as much as one ose (inoculating loop) put into 50 mL of BHI media and incubated on rotary shaker at 37°C for 24 hours. A 100 µL of *Staphylococcus aureus* inoculum was taken then suspended into 1 mL of BHI media, and incubated on rotary shaker at 37° C for 1 x 24 hours. Furthermore, the culture of *Staphylococcus aureus* was diluted using 0.9% NaCl sterile until the turbidity was equal to the McFarland 0.5 standard (1.5 x 10^8 CFU/mL).

Antibacterial Activity Test

The antibacterial activity test was conducted using the Kirby-Bauer method. A sterile cotton swab was inserted into the bacterial suspension and then evenly rubbed on the MHA surface media in a petri dish were dripped on a 6 mm blank disk paper therefore the disk contains 2 mg of extract or fractions. The disk paper containing extract and fraction was put aseptically on the MHA media that contained the test bacteria and then incubated at 37°C for 24 hours. The clear zone that formed around the disc indicates the sample can inhibit the growth of bacteria and the diameter can be determined.

Statistical Analysis

Raw data of total phenolic content and antibacterial effect were tested for normality and homogeneity. The normality test was calculated using the Kolmogorov-Smirnov and Lilliefors tests. Meanwhile, the homogeneity test was determined using the One Way ANOVA and LSD with a confidence interval (CI) of 95%. If the distribution was normal and homogeneous, then data were tested by One Way ANOVA with Tukey and LSD tests and the correlation test used the Pearson test. If the data were not normal nor homogenous, then the Kruskal-Wallis and Mann-Whitney tests were used.

RESULTS AND DISCUSSION

In this research, the extract of *Parsea americana* Mill. peels was prepared with the maceration method, which is a simple extraction method with a long extraction time. The extraction method could be used for thermolabile compounds. The ethanol was used as the extraction solvent because it is semi-polar solvent with a polarity index of 5.2 enabling the extraction of compounds with more distinct polarity (Zuraida *et al*., 2017). Ethanol is an effective solvent for extracting polyphenol compounds and is safe for human consumption. The ethanol has good extractability because it can penetrate cell walls easily (Zuraida *et al*., 2017).

The yield of *Parsea americana* Mill. extract with 96% ethanol solvent was 15.81%. The yield value obtained in this study is slightly different from the previous study results of 17.04% (Wulandari *et al*., 2019). The yield of the extract is closely related to the effectiveness of the extraction process which influenced by the type of solvent used, particle size, extraction method, and length of the extraction process (Salamah *et al*., 2017). Furthermore, the yield of extraction can be influenced by biological factors such as plant parts, plant species, harvesting time, and location of growth.

Fractionation is the process of separating a compound according to polarity level. The fractionation process was conducted sequentially, starting from the non-polar solvent to the polar solvent (Purwanto, 2015). The extract fractionation was done using *n*-hexane,

ethyl acetate, and methanol of separate compounds according to their degree of polarity. In this research, the yield of *n*-hexane, ethyl acetate, and methanol fraction was 27.41%, 8.34%, and 15.83%, respectively. The *n*-hexane can dissolve non-polar compounds such as lignin, wax, lipids, aglycons, sterols, and terpenoids. Ethyl acetate is effective to extract semi-polar compounds such as phenolic, aglycon, and glycoside compounds, and flavonoids. Methanol easily extracts various polar compounds, such as amino acids, sugar compounds and glycosides. In addition, phenolic compounds, especially those with low and medium molecular weight, and medium polarity are also easily soluble in methanol. (Widyawati *et al*., 2014).

Antibacterial activity of extract and fractions in *Parsea americana* Mill. peels are supported by phytochemical compounds such as total phenol compounds (Widyawati *et al*., 2014). The total phenolic content in both extracts and fractions was measured by the Folin-Ciocalteu method. In this method, the hydrogen atom in the phenolic compound is donated to the molybdenum ion contained in the Folin-Ciocalteau phenol reagent. The hydrogen donor stabilizes the phenoxyl radical by resonance or delocalization. The types and structural variations of phenolic compounds, as well as the number and position of the hydroxyl groups of the benzene ring affect the effectiveness of the reaction (Widyawati *et al*., 2014; Wong *et al*., 2016).

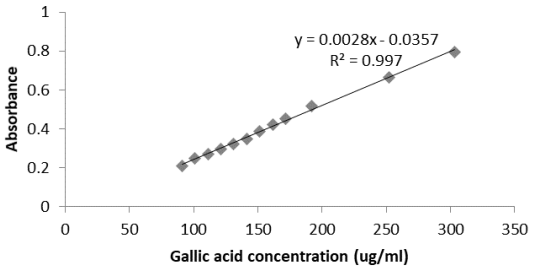

Figure 1. Calibration curve of standard gallic acid for determination of total phenolic content.

A linear calibration curve of gallic acid in range 90-300 ug/mL with a coefficient determination (r^2) value of 0.997 was obtained (Figure 1). The total phenolic compounds in the ethyl acetate fraction showed the highest concentration (536.26 ± 14.29 mg GAE/g) fraction compared to the other extract and fractions (Table 1). Ethanol extract, methanol and *n*-hexane fraction contained 148.72 ± 13.33 mg GAE/g extract, 76.44 ± 4.24 mg GAE/g fraction, and 43.94 ± 3.91 mg GAE/g fraction of total phenolic compounds. Based on the total phenolic content, the extract and fractions in *Parsea americana* Mill. peels had polar properties.

The total phenolic compounds of *Parsea americana* Mill. peels extract and fraction associated with the growth inhibition against *Staphylococcus aureus* ATCC 25923 were determined through antibacterial activity testing using the Kirby-Bauer method. The samples tested included ethanol extract, *n*-hexane, ethyl acetate, and the methanol fraction at a concentration of 10% w/v with the disks containing 2 mg of extract or fractions, negative control of 96% ethanol, and positive control of Vancomycin 30 µg. The results of the antibacterial activity of *Parsea americana* Mill. extract and fraction indicated the highest inhibition zone of 8.33 ± 0.58 mm (Figure 2) in the ethyl acetate samples, while the ethanol extract and methanol fractions were diameter of 5.67 ± 0.29 mm and 2.83 ± 0.29 mm, respectively. The *n*-hexane fraction did not show any inhibitory activity (Table 2). In the previous research, extract of *Parsea americana* Mill. contained phytochemical compounds, i.e. flavonoids, saponins, and alkaloids, which exhibited growth inhibition against *Staphylococcus aureus* ATCC 25923 (Wulandari *et al*., 2019).

Table 1. Total phenolic content of extract and fractions from *Parsea americana* Mill.

Sample	Replication (mg GAE*/g extract or fraction)			Average
	I	II	III	
Ethanol extract	141.68	137.14	155.35	144.73 ± 9.48
n-hexane fraction	39.71	44.69	47.43	43.94 ± 3.91
Ethyl acetate fraction	552.58	526.02	530.18	536.26 ± 14.29
Methanol fraction	72.18	76.50	80.65	76.44 ± 4.24

*GAE, Gallic acid equivalent.

Table 2. ***Inhibition zone diameter of the extract and fractions against Staphylococcus aureus***

Extract and Fractions	Inhibition Zone (mm)	Category
Ethanol Extract (10 % w/v, 20µL)	5.67±0.29	Weak
n-hexane Fraction (10 % w/v, 20µL)	0.00±0.00	-
Ethyl Acetate Fraction (10 % w/v, 20µL)	8.33±0.58	Medium
Methanol Fraction (10 % w/v, 20µL)	2.83±0.29	Weak
Negative Control (96% Ethanol, 20µL)	0,00±0,00	-
Positive Control (Vancomycin 30 µg)	12.17±0.29	Strong

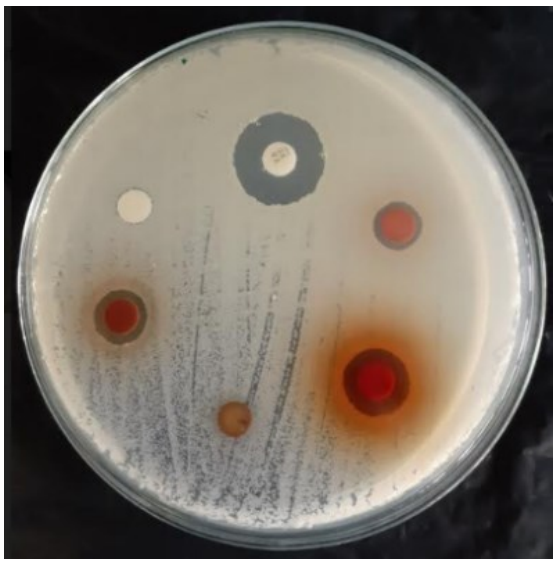

Figure 2. Screening of the active fraction; (a) ethanol extract, (b) *n*-hexane fraction, (c) ethyl acetate fraction, (d) methanol fraction, (e) positive control (Vancomycin 30 µg) and (f) negative control.

In the results of this study, the antibacterial activity of the extract and fractions were observed due to their phenolic content. The difference in the total phenolic of each sample affected the resulting zone of inhibition. This was proved by the finding that the higher the total phenolic content, the higher the zone inhibition diameter of *Staphylococcus aureus* ATCC 25923. The phenolic compounds will inhibit the growth of Gram-positive bacteria because of their ability to penetrate the bacterial cell walls (Purwantiningsih *et al*., 2014). Phenolic compounds reduce the permeability of the bacterial cell wall by destroying cell membranes, activating enzymes, and denaturing cell membrane proteins. Changes in the permeability of the cytoplasmic membrane can cause the transport of important organic ions into the cell to be disturbed. This will inhibit growth and even cause cell death. Studies have shown that high concentrations of phenolic compounds will penetrate and disrupt bacterial cell walls (Purwantiningsih *et al*., 2014).

An antibacterial agent has activity against bacteria if it has the strength as follows: when the inhibition zone has a size of less than 5 mm, it is categorized as weak, 5-10 mm is categorized as moderate, 11-20 mm is categorized as strong, and more than 20 mm means the activity is very strong (Rahmawati *et al*., 2014). Based on this, the ethyl acetate fractions at a concentration of 10% w/v which produced an inhibition zone of 8.33 ± 0.577 mm is included in the medium category.

The results of the statistical analysis show that antibacterial activity data at extract and fractions of *Parsea americana* Mill. were not normally distributed and not homogeneous. Based on the normality test of antibacterial activity with $p>0.05$ in the Kolmogorov-Smirnov and Lilliefors tests, the result was 0.200 ($p>0.05$) and then homogeneity test was 0.012 ($p<0.05$) using One Way ANOVA. Accordingly, it was necessary to test the data using the Kruskal-Wallis and Mann-Whitney tests. In the Kruskal-Wallis test, the result was 0.005 ($p<0.05$). The Mann-Whitney test showed a significant difference between each extract and fraction except there was no significant difference in the *n*-hexane fraction with ethanol 96% as a negative control. The results of the statistical analysis shows that total phenolic content data were normally distributed and homogeneous with 0.200 ($p>0.05$) in the Kolmogorov-Smirnov and Lilliefors tests and 0.082 ($p>0.05$) using One Way ANOVA. The LSD and Tukey HSD analysis showed $p<0.05$, indicating there was significant difference in total phenol content between the extract and each fraction tested. The correlation test using the Pearson test showed 0.000 ($p<0.05$). The correlation coefficient value was 0.867 which means there is a correlation between the total phenolic content and antibacterial activity and the value is in the range 0.81-1.00 indicating a very strong positive correlation where the higher the total phenolic content of extract and fractions, the greater the inhibitory zone against *Staphylococcus aureus* bacteria.

CONCLUSIONS

Inhibitory activity of *Parsea americana* Mill. peels extract and fractions against *Staphylococcus aureus* showed that the ethyl acetate fraction had the highest total phenolic content with 536.26 ± 14.29 mg GAE/g fraction and the inhibition zone was 8.33 ± 0.58 mm compared to the other fractions and extract.

ACKNOWLEDGMENTS

Thanks to The Ministry of Education and Culture for the Master's Thesis Research grant 2020.

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